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How Do Lignin Composition, Structure, and Cross-Linking Affect Degradability? A Review of Cell Wall Model Studies

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ABSTRACT

Because of the complexity of plant cell wall biosynthesis, the mechanisms by which lignin restrict fiber degradation are poorly understood. Many aspects of grass cell wall lignification and degradation are successfully modeled by dehydrogenation polymer-cell wall (DHP-CW) complexes formed with primary walls of corn *Zea mays* L. This system was used to assess how variations in lignin composition, structure, and cross-linking influence the hydrolysis of cell walls by fungal enzymes. Altering the normal guaiacyl, syringyl, and *p*-hydroxyphenyl makeup of lignin did not influence cell wall degradability; each unit of lignin depressed cell wall degradability by two units. Plants with perturbed lignin biosynthesis often incorporate unusual precursors into lignin and one of these, coniferaldehyde, increased lignin hydrophobicity and further depressed degradability by up to 30%. In other studies, lignin formed by gradual "bulk" or rapid "end-wise" polymerization of monolignols had markedly different structures but similar effects on degradability. Reductions in cell wall cross-linking, via oxidative coupling of feruloylated xylans to lignin or nucleophilic addition of cell wall sugars to lignin quinone-methide intermediates, increased the initial hydrolysis of cell walls by up to 46% and the extent of hydrolysis by up to 28%. Overall, these studies suggest that reductions in lignin concentration, hydrophobicity, and cross-linking will improve the enzymatic hydrolysis and utilization of structural polysaccharides for nutritional and industrial purposes. In ongoing work, we are developing a DHP-CW system for dicots and are investigating how cross-linking and various acylated and unusual monolignols influence the formation of lignin and the degradation of cell walls by rumen microflora.

Lignin plays a vital role in plant growth and development by improving water conduction through xylem tracheary elements, enhancing the strength of fibrous tissues, and limiting the spread of pathogens in plant tissues (Iiyama et al., 1994). Lignin restricts the degradation of structural polysaccharides by hydrolytic enzymes, thereby limiting the bioconversion of forages and fibrous crops into animal products or into liquid fuels and other industrial products (Brown, 1985; Jung and Deetz, 1993). Lignified dietary fiber also plays an important role in maintaining gastrointestinal function and health in humans (Ferguson et al., 2001).

The enzymatic degradability of cell walls in leaves and particularly stems of plants declines during maturation because of accumulation and progressive lignification of primary and secondary cell walls of vascular and sclerenchyma tissues. As plants reach physiological maturity, the degradability of stems, and to a lesser degree leaves, is further depressed by lignification of primary-walled parenchyma and epidermal tissues (Wilson and Hatfield, 1997). These reductions in degradability are partly related to the increased lignin content of cell walls; however, variations in three-dimensional structure and composition of lignin and its hydrophobicity, encrustation, and cross-linking to other matrix components also have been implicated (Chesson, 1993; Jung and Deetz, 1993).

Because of the anatomical, morphological, and developmental complexity of cell wall and lignin formation in various plant tissues, studies with normal, mutant, or transgenic germplasm often do not show consistent relationships between lignin-matrix characteristics and cell wall degradability at the whole plant, plant part, or even tissue level (Grabber et al., 1992; Jung and Vogel, 1992; Jung et al., 2000). Even when plant selection or enzyme downregulation is targeted at specific lignin properties or lignin-matrix interactions, compensatory or associative changes in other cell wall characteristics often occur, making it difficult to identify underlying mechanisms controlling cell wall degradability. Plants may, for example, respond to lower lignification by increasing the amount of cross-linking (Chabannes et al., 2001), perhaps yielding no net change in digestibility. A common but faulty assumption is that correlations between a particular lignin trait and degradability demonstrate a cause and effect relationship. Because of the complexity of cell wall development, associations between one lignin characteristic (e.g., lignin composition) and cell wall degradability can be masked, confounded, or merely correlated to concurrent changes in other lignin properties (e.g., lignin cross-linking) that influence cell wall degradability. Because of these factors, the underlying mechanisms by which lignin restricts degradability of plant cell walls are poorly understood.

An Overview of Cell Wall Model Systems

Because of the developmental and morphological complexity of plants, a variety of simpler model systems have been developed in an attempt to isolate and identify potential interactions between lignin and matrix components and, in some cases, to assess how these interactions influence cell wall hydrolysis by enzymes or rumen microflora. One approach involves adding, removing, or modifying lignins or other matrix components in isolated cell walls or cellulose by chemical or enzymatic treatments to assess their role in controlling

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; DHP, dehydrogenation polymer; DHP-CW, dehydrogenation polymer-cell wall; G, guaiacyl; H, *p*-hydroxyphenyl; NMR, nuclear magnetic resonance; PME, pectin methyl esterase; S, syringyl.

cell wall degradability. The simplest studies (Jung and Ralph, 1990; Sewalt et al., 1996a) involved adding powdered wood lignin or an artificial lignin (polyeugenol) to cellulose before hydrolysis by rumen microflora. More intimate associations between components were obtained by forming lignocellulosic hydrogels or by nonspecific coupling of polyeugenol to cellulose. These studies yielded contradictory results with cellulose hydrolysis being inhibited by added wood lignins but not by added polyeugenol. In contrast, bound polyeugenol severely restricted cellulose hydrolysis whereas intimate association of lignin with cellulose in hydrogels actually enhanced cellulose hydrolysis. Various chemical or biological treatments have been used to remove or modify lignins and to cleave lignin-matrix cross-links in cell walls (Fritz et al., 1991; Jung et al., 1992; Morrison, 1991), but the specificity of these treatments was poor (Fry, 1986), making it difficult to attribute changes in degradability to specific changes in cell wall properties. In contrast, enzymes like cinnamoyl esterases can attack specific linkages found in cell walls (Garcia-Conesa et al., 1999a), but their efficacy *within* lignified cell walls remains unproven. Alternatively, the activity of many enzymes involved in cell wall biosynthesis can be manipulated in plants by inhibitors and elicitors (Grand et al., 1985; Reddy et al., 1999). Unfortunately, the utility of elicitors or inhibitors in cell wall degradability studies would be limited because they often lead to multiple changes in cell wall characteristics.

Plant tissue culture also can be used to model cell wall formation and lignification, the most successful system being the differentiation of up to 60% of *Zinnia elegans* L. mesophyll cells into tracheary elements (Fukuda, 1992). This system has been extremely valuable for cell biology and gene expression studies of vascular differentiation, secondary wall formation, and lignification (Milioni et al., 2001). Unfortunately, each *Zinnia* culture produces only a few milligrams of cell walls, limiting its application in cell wall degradability studies. Gram quantities of cell walls can be produced with other tissue culture systems, but the degree of differentiation and lignification is typically much lower, except perhaps for cell cultures with high endogenous levels of cytokinin (Blee et al., 2001). In all cases, differentiating cultures produce a mixture of primary- and secondary-walled cell types with nonlignified cells providing phytohormones, monolignols, or other factors needed by differentiating cells for secondary wall formation and lignification (Hosokawa et al., 2001). As a result, characterization of specific cell types in differentiated culture systems would require the same type of fractionation procedures as used to isolate tissues from intact plants (Grabber and Jung, 1991; Hatfield et al., 1999). To a greater degree than with plants, cell walls from cultured cells can be manipulated to varying degrees by chemical, enzymatic, biological, elicitor, or enzyme inhibitor treatments (Kaliamoorthy and Krishnamurthy, 1998; Kauss et al., 1993; Taylor and Haigler, 1993) or by altered gene expression (Stasolla et al., 2003), but specific alteration of individual lignin characteristics without compensatory changes in other lignin-matrix interactions would be difficult to obtain. In addition, interactions between matrix components and lignin in cell culture systems, as with plants, are difficult to quantify because the deposition of matrix components and lignin are often overlapping processes during cell wall formation. This difficulty is further compounded by our inability to fully isolate or characterize, by solvolytic or spectroscopic methods, lignin and lignin-matrix structures in cell walls. The prospects for lignin and cell wall characterization have, however, improved considerably with the recent discovery of solvent systems capable of fully dissolving ball-milled cell walls for analysis by NMR and other analytical methods (Lu and Ralph, 2003).

Another approach, often enabling specific alteration of individual lignin characteristics or lignin-matrix interactions, is the in vitro oxidative polymerization of lignin precursors (i.e., monolignols) into DHPs, either alone or in the presence of natural or artificially synthesized cell wall components (Higuchi et al., 1971; McDougall et al., 1996; Ohnishi et al., 1992; Ralph et al., 1992; Terashima et al., 1995; Touzel et al., 2003). In this system, a wide variety of monolignol types, matrix components, and polymerization conditions may be used, providing the investigator with a much greater control over the composition and structure of lignin and lignin-matrix interactions formed than is possible with living plant systems. Characterization of matrix components and monolignols before and after DHP formation permits the characterization of lignin-matrix interactions to a degree that is not possible in natural plant systems where matrix components and lignin are often deposited concurrently into composite structures that are difficult to analyze by spectroscopic or solvolytic methods. Although this in vitro system has been extremely valuable for modeling many aspects of cell wall lignification, artificial DHP lignins do not fully mimic the structure of natural plant lignins (Terashima et al., 1996), and they do not adequately model the three-dimensional matrix of cell walls.

The limitations of the tissue culture and DHP model systems are largely overcome by using in situ peroxidases to polymerize monolignols added to isolated cell walls. In the late 1970s, Whitmore (1978) was the first to form such DHP-cell wall complexes to study lignin-protein and lignin-carbohydrate interactions in cell walls isolated from callus of *Pinus elliottii* Engelm. Since the early 1990s, our objective has been to develop, evaluate, and utilize DHP-CW complexes as a model for studying lignin formation and lignin-matrix interactions in plants and for assessing how lignins restrict the enzymatic degradation of cell walls.

Formation and Characteristics of Grass DHP-CWs

Thus far, we have formed DHP-CW complexes with primary cell walls isolated from nonlignified cell suspensions of maize (cv. Black Mexican) (Grabber et al., 1996b). Cell walls from these suspensions were composed of 194 g kg^{-1} of arabinose, 169 g kg^{-1} of xylose, 78 g kg^{-1} of galactose, 316 g kg^{-1} of glucose, 9 g kg^{-1} of rhamnose, 8 g kg^{-1} of mannose, 126 g kg^{-1} of uronic acids, 9 g kg^{-1} of

methanol, 20 g kg^{-1} of ester-linked ferulate and diferulates, 0.4 g kg^{-1} of *p*-coumarate, 0.5 g kg^{-1} of ether-linked ferulate and diferulates, 3 g kg^{-1} of guaiacyl lignin, and 30 g kg^{-1} of protein (Grabber et al., 1998, 2003, 1995). Overall, the composition of cell walls from maize cell suspensions was representative of non-lignified primary walls of grasses (Carpita, 1996; Carpita et al., 2001). As illustrated below, the composition or structure of cell walls used to form DHP-CWs can be manipulated by growing cell cultures with enzyme inhibitors or elicitors or by treating isolated cell walls with enzymes or chemicals.

As with other plant species, maize cells secrete peroxidases, so trying to lignify walls by adding monolignols to the culture medium would result in the formation of DHPs in solution (Brunow et al., 1993) or on the cell wall surface (Nakashima et al., 1992) rather than within the wall matrix. To minimize this, cells are ruptured and extracted with buffer and aqueous $CaCl_2$ to remove cytoplasmic and loosely bound apoplastic peroxidases from cell walls before artificial lignification. Walls isolated in this manner contain an array of peroxidase isozymes with substrate affinities similar to those identified in lignifying maize plants (Grabber et al., 1996b). Dehydrogenation polymer-cell wall complexes are then usually formed by suspending isolated cell walls in 10 m*M* Homopipes buffer (pH 5.5) and adding, via a peristaltic pump, separate solutions of monolignols and hydrogen peroxide over a 24-h period. As an alternative, glucose may be added with monolignols to a cell wall suspension that contains glucose oxidase to generate hydrogen peroxide in situ. The quantity, type, or sequence of monolignols added, their rate of addition, and the pH of the reaction medium can all be varied to manipulate the formation of lignin and its interactions with other cell wall components. After DHP-CW formation is complete, cell walls are thoroughly washed with water followed by acetone to remove nonbound lignins.

In most cases, incorporation of monolignols into wall-bound DHPs usually exceeds 90% and DHP-CWs with Klason lignin concentrations approaching 300 g kg^{-1} can be formed (J.H. Grabber, unpublished data). Klason lignin concentrations, however, are limited to about 140 g kg^{-1} if cell walls are lignified very rapidly (<2 h), lignified under acidic conditions (pH < 4.5), or lignified with a high proportion of sinapyl alcohol (>65%). With rapid lignification, DHPs are readily formed but about one-half are readily extracted from DHP-CWs with acetone because they are not bound to cell walls. Under low pH conditions, low yields are caused by accelerated inactivation of cell wall peroxidase. Low yields of syringyl-rich lignins are due to the low affinity of maize peroxidases for sinapyl alcohol and to the propensity of sinapyl alcohol to undergo dimerization rather than polymerization.

Structural analysis of DHP-CWs by thioacidolysis, pyrolysis, and NMR indicate that synthetic lignins formed in this system are quite comparable to naturally formed grass lignins (Grabber et al., 1996b). Like naturally lignified grass cell walls, DHPs are distributed throughout the wall matrix, they have comparable effects in depressing the enzymatic hydrolysis of structural polysaccharides, and about one-half are released from cell walls by aqueous alkaline solutions.

Since DHP-CWs are formed with primary maize walls, this system should most accurately model the lignification and enzymatic degradation of primary walled parenchyma and epidermal tissues in grasses. However, our system may also adequately model secondary walled xylem and sclerenchyma tissues in maturing grasses because primary walls in these tissues tend to be the most heavily lignified and resistant to degradation and they form a barrier limiting the degradation of adjacent tissues that are not directly exposed to rumen microflora or hydrolytic enzymes (Chesson et al., 1986; Engels and Schuurmans, 1992; Wilson and Hatfield, 1997). Furthermore, in mature grass stems the structural carbohydrate composition, lignin content, and degradability of primary and secondary walled tissues are, in many respects, quite similar (Grabber et al., 1991; Hatfield et al., 1999).

Because of the structural similarities between DHP-CW and natural plant lignins, and the importance of primary cell walls in controlling wall properties in grasses, this system is useful for studying the formation and characteristics of lignin-matrix interactions in grass cell walls. As illustrated below, a wide array of lignin-matrix interactions can be manipulated in DHP-CWs to examine their effect on enzymatic hydrolysis of structural polysaccharides, making this system valuable for identifying targets for genetic modification of plants to improving the utilization of lignocellulosic materials as feedstuffs for livestock and as feedstocks for bioconversion into fuels and other products. Finally, DHP-CWs could be valuable in small animal or in vitro studies aimed at elucidating the beneficial effects of lignified fiber on gastrointestinal function and health.

Model Studies of Grass Cell Wall Lignification and Degradability

Monolignol Composition

Lignins in forages are composed primarily of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. At early stages of lignification, coniferyl alcohol with small amounts of *p*-coumaryl alcohols is copolymerized into the primary wall to form mixed G and H lignins. Later during secondary wall development, coniferyl alcohol and increasing amounts of sinapyl alcohol are copolymerized to form mixed G and S lignins (He and Terashima, 1990, 1991). Digestibility was reported to be negatively, positively, or not related to the concentration or ratio of S and G units in lignin (Baucher et al., 1999; Jung and Casler, 1991; Jung et al., 1994, 1999; Jung and Buxton, 1994). Modifications to the S to G ratio of lignins, because of plant selection or direct gene manipulation, often elicit the incorporation of unusual monolignols into lignin and alter the concentrations of lignin, *p*-coumarate esters, ferulate cross-links, and perhaps other cell wall components (Chabbert et al., 1994; Jung and Casler, 1991; Lam et al., 1996; Marita et al., 2003; Thorstensson et al., 1992; Vailhe et al., 1998). The distribution of cell wall phenolics and plant anatomy may

also be affected (Goto et al., 1993; Grenet and Barry, 1991; Morrison et al., 1993). Because of these and possibly other confounding effects, it has not been demonstrated whether normal variations in lignin composition directly affect the enzymatic degradability of cell walls.

In our model studies (Grabber et al., 1997), wall-bound DHPs were formed with coniferyl alcohol alone or in 1:1 ratios with *p*-coumaryl and sinapyl alcohols (Fig. 1, Table 1). Dehydrogenation polymer-cell wall complexes with G lignin and with mixed H and G lignin were efficiently formed with Klason lignin concentrations often exceeding predicted values because of covalent attachment of ferulates, protein, or carbohydrate to lignin (Grabber et al., 1996b). The various DHP-CWs had similar amounts of ferulates (16 g kg^{-1}) coupled to lignin and similar alkaline-solubility of lignin (~50%), suggesting that lignin composition did not appreciably alter interactions between lignin and other matrix components. Incubation of DHP-CWs with fungal enzymes revealed that lignins formed with G units, H and G units, or S and G units depressed structural polysaccharide degradation to the same degree. This suggests that improvements in fiber degradability, previously attributed to modifications in lignin composition of mutant and transgenic germplasm, were probably due to other associated changes in wall chemistry or ultrastructure. Although the proportions of H, G, and S units in lignin are probably not a direct factor controlling degradability, reduced deposition of one or more of these units can enhance degradability if the overall concentration and distribution of lignin is also reduced. In ongoing work, we will assess how wider shifts in lignin composition, ranging from 100% G to 100% S units, influence the degradability of DHP-CWs by rumen microflora.

In addition to normal H, G, and S units, lignins can contain high levels of unusual phenylpropane units, particularly if enzymes in the lignin pathway are perturbed by mutations or genetic engineering (Boerjan et al.,

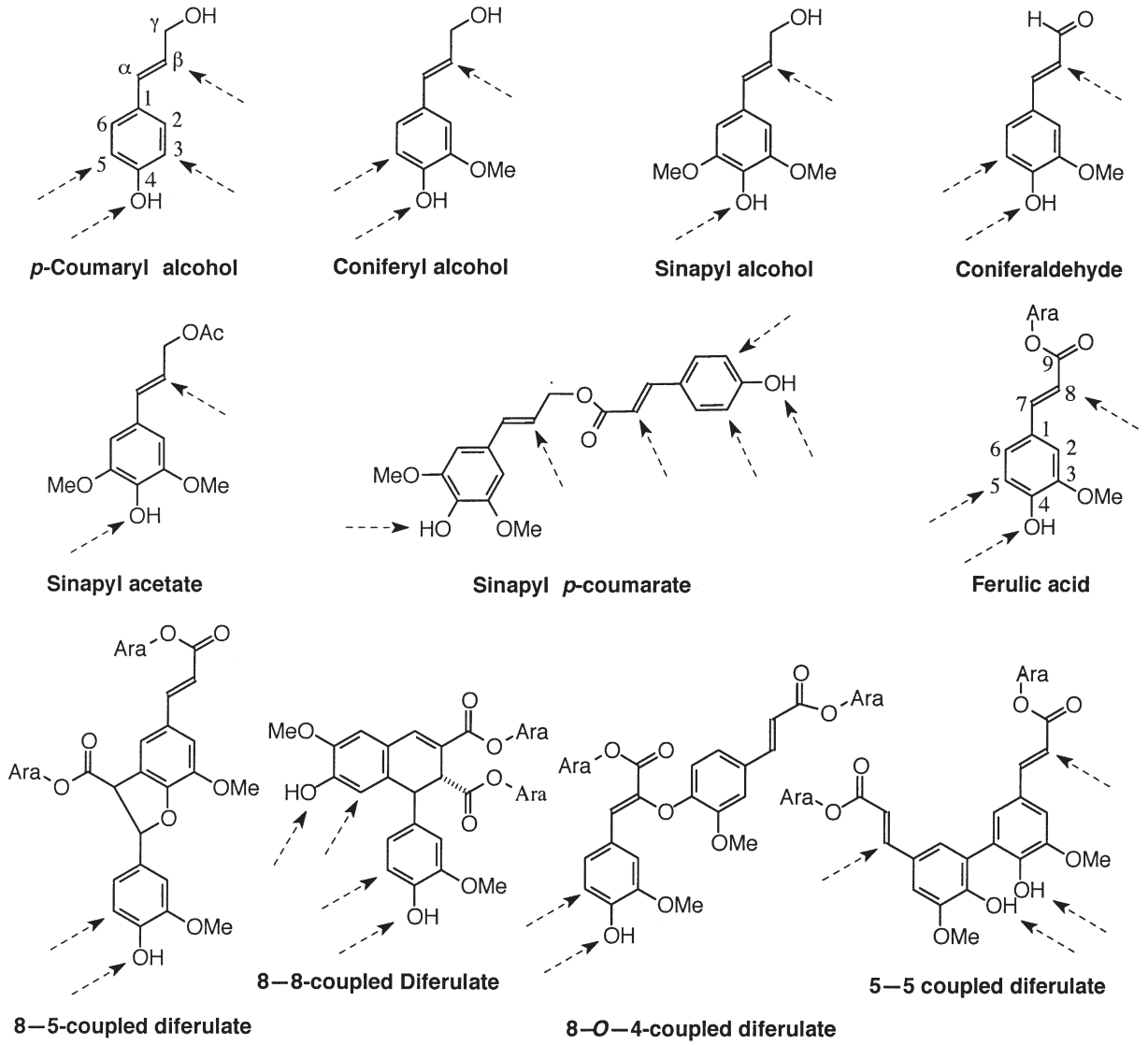

Fig. 1. Lignin precursors (i.e., monolignols) used to form DHP-CWs with primary maize walls and major ferulate and diferulate esters forming xylan–xylan and xylan–lignin cross-linked structures in DHP–CWs. 'Ara' are arabinofuranosyl residues on arabinoxylans. Dotted arrows indicate potential sites for coupling with monolignols during lignin polymerization.

Table 1. Change in the amount of sugars released from DHP-CWs by fungal enzymes in response to shifts in lignin composition, structure, or cross-linking.†

Lignin characteristic	Klason lignin‡	Change in sugars released§	
		6 h	72 h
Shift in composition			
Guaiacyl to guaiacyl:*p*-hydroxyphenyl units (1:1)	108	−9 (−4%)	−21 (−4%)
Guaiacyl to guaiacyl:syringyl units (1:1)	108	0 (0%)	−2 (−0.4%)
Guaiacyl to guaiacyl:coniferaldehyde units (1:1)	138	−68 (−32%)*	−70 (−15%)*
Guaiacyl to coniferaldehyde units	138	−97 (−46%)*	−137 (−29%)*
Shift in structure			
Branched "bulk" to linear "end-wise" polymers	120	5 (3%)	−2 (−0.5%)
Shift in cross-linking			
Ferulate cross-linking reduced from 15.9 to 4.5 g kg^{-1}¶	153	79 (46%)*	82 (20%)*
Ferulate cross-linking reduced from 15.8 to 5.2 g kg^{-1}#	103	51 (24%)*	114 (25%)*
Benzyl ether and ester cross-linking reduced††	120	49 (38%)*	104 (28%)*

* $P < 0.05$.
† From Grabber et al. (1997, 1998b, 1998c, 2003).
‡ Average Klason lignin content (g kg^{-1}) of treatments.
§ Absolute (g kg^{-1}) and relative (%) change in the amount of sugars released from DHP-CWs because of shifts in lignin characteristics. DHP-CWs were degraded for 6 and 72 h with a mixture of Celluclast and Viscozyme. In some studies, the release of sugars was adjusted by covariant analysis to account for differences in the Klason lignin content of DHP-CWs.
¶ Ferulate cross-linking in DHP-CWs was reduced by growing maize cell cultures with a phenylalanine ammonia lyase inhibitor before artifical lignification of isolated cell walls.
# Ferulate cross-linking in DHP-CWs was reduced by methylating ferulates with diazomethane before artificial lignification of isolated cell walls.
†† Benzyl ether and ester cross-linking was reduced by increasing the pH during lignification from 4.0 to 5.5. Quantities of cross-links formed were not determined.

2003). Plants deficient in cinnamyl alcohol dehydrogenase (CAD) activity are of considerable interest because their cell-wall degradability can be up to 50% greater than their normal counterparts (Baucher et al., 1999; Provan et al., 1997; Thorstensson et al., 1992; Vailhe et al., 1998). Lignin in CAD deficient plants often contains elevated levels of *p*-hydroxycinnamaldehyde units, which is not surprising since this enzyme catalyses the reduction *p*-hydroxycinnamaldehydes to their corresponding alcohols. Other modifications that accompany CAD deficiency include reduced levels of *p*-coumarate esters and ferulate ethers, reduced deposition of S or G lignins, and increased deposition of unusual phenylpropane units or aldehydes into lignin or cell walls (Kim et al., 2003; Provan et al., 1997; Vailhe et al., 1998).

In model studies, coniferaldehyde (Fig. 1) was readily incorporated into wall-bound DHPs as the sole monolignol or in mixtures with coniferyl and sinapyl alcohols (Grabber et al., 1998b; J.H. Grabber, unpublished data). Although all DHP-CWs had similar high levels of ferulate-lignin cross-linking, the alkaline solubility of lignins formed with coniferaldehyde was >90%, compared with about 50% for lignins formed with coniferyl alcohol. Cleavage of ferulate ester cross-links contributes to the alkaline solubility of both types of lignin, but, unlike normal lignins, coniferaldehyde lignins have high solubility because they do not form alkali-stable benzyl ether cross-links with structural polysaccharides via quinone methide intermediates (Grabber et al., 1998b). Although coniferaldehyde lignins formed fewer cross-linked structures with other wall components, they were much more inhibitory to cell wall degradation than lignins formed with coniferyl alcohol (Table 1). Degradability differences were diminished if enzyme loadings were increased and if hydrophobic aldehyde groups in lignins were reduced to their corresponding alcohols by reduction with ethanolic sodium borohydride. Increased hydrophobicity of coniferaldehyde lignins probably exacerbates binding of cell wall degrading enzymes to lignin and restricts hydration and penetration of these enzymes into cell walls (Sewalt et al., 1997). Our studies suggest that improved enzymatic degradability of CAD-deficient plants is not due to the accumulation of aldehyde-enriched lignins. In fact, highly hydrophobic aldehyde lignins caused a severe depression in cell wall degradability in a model system where other cell wall properties remained constant. Therefore, improved degradation of CAD-deficient plants must be attributed to other associative changes in cell wall composition and structure. Although incorporation of aldehyde units into cell walls is not desirable from a nutritional standpoint, their inhibitory effects on cell wall degradability and possible biocidal properties may provide additional protection against plant pests. The high alkaline solubility of these lignins should also enhance delignification of plants for paper production and for fermentation into ethanol and other chemicals. In ongoing work, we are assessing how high levels of other unusual monolignols (coniferaldehyde 5-hydroxyconiferyl alcohol, dihydroconiferyl alcohol, etc.) observed in plants with perturbed lignin biosynthesis (Kim et al., 2003; Marita et al., 2003; Piquemal et al., 2002) influence the degradability of DHP-CWs by rumen microorganisms.

Monolignol Acylation with *p*-Coumarate and Acetate

Lignins in grasses are acylated with *p*-coumarate, which is primarily attached to the γ−position of S units (Grabber et al., 1996a; Ralph et al., 1994). Although very small quantities of *p*-coumarate are esterified to arabinoxylans in immature tissues, most *p*-coumarate accretion occurs in tandem with lignification, making *p*-coumarate accumulation a convenient indicator of lignin deposition in grasses (Morrison et al., 1998; Musel et al., 1997; Vailhe et al., 2000). Concentrations of *p*-coumarate vary considerably among species at maturity but they tend to be higher in C4 than in C3 grasses (Cherney et al., 1989; Ford and Elliott, 1987; Grabber et al., 1991).

On the basis of the analysis of isolated lignins and cell walls, *p*-coumarate can comprise up to 20% of the lignin in maize and sorghum [*Sorghum bicolor* (L.) Moench] tissues (Hatfield et al., 1999; Morrison et al., 1998; Ralph et al., 1994). Unlike ferulate polysaccharide esters, *p*-coumarate esters on lignin do not extensively participate in oxidative coupling reactions, and most remain as terminal units with a free phenolic group and an unsaturated side chain (Ralph et al., 1994). High levels of *p*-coumarate etherified to lignin have been reported for grasses, but this may in part arise because of difficulties in fully hydrolyzing *p*-coumarate esters in lignified walls (Hartley and Morrison, 1991). Although *p*-coumarate usually forms few cross-linked structures by radical coupling mechanisms it can, however, undergo photocatalyzed cyclodimerization to form truxillic and truxinic acids (Hartley and Morrison, 1991).

Syringyl-rich lignins of kenaf (*Hibiscus cannabinus* L.) are extensively γ-acylated (>50%) with acetate (Ralph, 1996; Ralph and Lu, 1998). Natural acetylation of lignin may occur in other plant species but has probably been missed because lignins are typically preacetylated before NMR analysis. Minor acylation of some hardwood lignins with acetate and *p*-hydroxybenzoylates has also been reported (Ralph and Lu, 1998).

Some structural evidence suggests that sinapyl alcohol is enzymatically acylated with acetate or *p*-coumarate before lignin polymerization (Lu and Ralph, 1999, 2002). The function of lignin acylation is unknown but in vitro studies suggests that transfer of radicals from *p*-coumarate to sinapyl alcohol may aid in the formation of syringyl-rich lignin in some plant species (Takahama et al., 1996). In recent studies (J.H. Grabber, unpublished data), the formation of syringyl-rich lignins in cell walls was increased by 20 g kg^{-1} if the monolignol mixture included sinapyl acetate (Fig. 1). A similar increase in syringyl-rich lignin deposition was observed if cell walls were lignified with moderate amounts (~100 g kg^{-1}) of a monolignol mixture that included sinapyl *p*-coumarate (Fig. 1). At higher addition rates, this mixture tended to decrease lignin deposition because of accelerated inactivation of wall-bound peroxidase by sinapyl *p*-coumarate. Adding sinapyl *p*-coumarate to the monolignol mixture also increased the amount of alkalilabile ferulates recovered from DHP-CWs, suggesting that *p*-coumarate esters impaired the copolymerization of ferulates with monolignols. We are currently evaluating the effects of lignin acylation with acetate and *p*-coumarate on cell wall degradability.

Lignin Structure

The rate of monolignol secretion into the apoplast influences the structure of lignin and possibly its interactions with other matrix components. Rapid "bulk" polymerization, as may occur in the middle lamella and primary wall, favors C–C coupling of monolignols into a highly branched polymer. In contrast, gradual "end-wise" polymerization, as may occur in secondary walls, favors β–*O*–4 coupling of monolignols into a relatively linear polymer (Jurasek, 1998). Because the degradability of primary walls in lignified grass tissues is often considerably less than secondary walls it has been proposed that highly branched lignins are more inhibitory to cell wall degradability (Jung and Deetz, 1993).

In initial studies, DHP-CWs were formed by gradual "end-wise" or rapid "bulk" polymerization of coniferyl alcohol, added at rates of up to 350 g kg^{-1} of cell wall (Grabber et al., 2003). The yield of guaiacyl-lignin derived monomers recovered after thioacidolysis of end-wise polymers was about 2.2 times greater than that of bulk polymers, indicating that the former had a much greater proportion of β-*O*-4 ether linkages. A high proportion of β-*O*-4 ether linkages are characteristic of relatively linear lignin polymers. As with naturally formed lignins (Terashima et al., 1996), end-wise DHP-CWs also had a much lower proportion of ether-linked coniferyl alcohol end-groups (~9%) compared with bulk DHP-CWs (~27%). End-groups are abundant under bulk polymerization conditions, which favor coniferyl alcohol dimerization rather than end-wise addition of coniferyl alcohol monomers to a growing lignin polymer. In contrast, DHPs formed in solution contain a high proportion of end groups (15–40%) regardless of whether they are formed under bulk or end-wise polymerization conditions (Terashima et al., 1996). The markedly differing levels of end-groups, in addition to substantial differences in thioacidolysis yields, provides compelling evidence that end-wise and bulk polymers in DHP-CWs had vastly different structures. Although both types of polymers were readily formed, incorporation of end-wise polymers into cell walls exceeded 90%, while incorporation of bulk polymers declined to 45% at the highest rate of monolignol addition. Maximal Klason lignin concentrations of DHP-CWs was 270 g kg^{-1} for endwise polymers compared to only 165 g kg^{-1} for bulk polymers. Incorporation of ferulates and diferulates was essentially complete when DHP-CWs reached Klason lignin concentrations of about 150 g kg^{-1}. Ferulates probably act as nucleation sites for lignin formation and their extensive copolymerization with monolignols helps to anchor lignins into cell walls during the early stages of lignification (Grabber et al., 2002a); therefore, depletion of noncoupled ferulates may be one reason lignification was less efficient under bulk polymerization conditions. Once ferulate and diferulates are incorporated, further lignification under bulk polymerization conditions would essentially stop because oxidative coupling of newly formed dilignols or oligomers to wall bound polymers is very inefficient (Sarkanen, 1971). In the case of end-wise polymerization, cell wall lignification continues even after ferulate incorporation is complete because monolignols readily couple to growing lignin polymers (Sarkanen, 1971). Therefore, if monolignol polymerization in grasses begins as a bulk process, it must quickly transition to an end-wise process to permit extensive lignification of primary cell walls, particularly in highly lignified tissues like xylem vessels and sclerenchyma.

Adjusting data to account for differences in lignin content revealed that lignins of divergent structure, formed by bulk and end-wise polymerization, had simi-

lar effects on DHP-CW degradability (Table 1). The latter findings are consistent with our earlier work demonstrating that artificial lignins formed with varying ratios of *p*-coumaryl, coniferyl, and sinapyl alcohols have a similar impact on cell wall degradability (Grabber et al., 1997), even though coniferyl and possibly *p*-coumaryl alcohols form more highly branched lignins than sinapyl alcohol (Saake et al., 1996). In future studies, our group will try to determine whether lignins formed in the apoplastic space, but not cross-linked to matrix components, limit cell wall degradability.

Ferulate and Diferulate Cross-Linking

Ferulates are intracellularly esterified to the C5-hydroxyl of α-L-arabinose sidechains of xylans and deposited into primary and secondary walls of a variety of grass tissues (Jung, 2003; MacAdam and Grabber, 2002; Migne et al., 1998; Myton and Fry, 1994). During cell wall deposition and lignification, xylans are cross-linked by peroxidase-mediated coupling of ferulate monomers into a complex array of dimers and trimers and by extensive copolymerization of these ferulates into lignin (see review by Grabber et al., 2004). Oxidative coupling of ferulates probably contributes to wall stiffening, lignin formation, growth cessation, and pest resistance of plants (Bergvinson et al., 1997; Bily et al., 2003; Grabber et al., 2002a; MacAdam and Grabber, 2002; Schopfer, 1996), and it influences the physical properties of foods (Oosterveld et al., 1997; Schooneveld-Bergmans et al., 1999). Ferulates and diferulates also have properties that may be beneficial to human health (Ferguson et al., 2003; Garcia-Conesa et al., 1999b).

Ferulate substitution, diferulate cross-linking of xylans, and ferulate cross-linking of xylans to lignin were long thought to structurally impede the enzymatic hydrolysis of grass walls (Hartley, 1972), but consistent relationships have not been observed in studies with normal plant populations or mutant germplasm (Goto et al., 1994; Jung and Casler, 1991; Jung and Vogel, 1992; Jung and Buxton, 1994; Lam et al., 1996, 2003; Mechin et al., 2000). Correlative studies such as these are often hampered because the quantity of ferulates recovered after alkaline or acid hydrolysis represent only a small and variable proportion of the total ferulates deposited in cell walls (Grabber et al., 2004). In addition, associations between ferulate deposition and cross-linking with cell wall degradability are probably masked or confounded by concurrent changes in other wall components that normally occur during plant development or that are brought about by genetic manipulation of cell wall biosynthesis. As discussed previously (Grabber et al., 1998a), initial attempts to model or isolate the effects of ferulates on cell wall degradability by artificial feruloylation of fiber or treatment with ester-cleaving chemicals and enzymes suffered from a lack of realism, specificity, or effectiveness.

In our studies (Grabber et al., 1998a), nonlignified cell suspensions of maize were grown with or without 2-aminoindan-2-phosphonic acid (an inhibitor of phenylalanine ammonia lyase) to produce cell walls with reduced (5.1 g kg^{-1}) or normal (17.2 g kg^{-1}) concentrations of ferulate monomers and dimers (Fig. 1). Isolated walls were then incubated with mercaptoethanol to inhibit further diferulate formation or with hydrogen peroxide to stimulate ferulate dimerization by wall bound peroxidases. Simply changing the degree of cell wall feruloylation had little effect on degradability, but increasing the proportion of ferulate dimers from 20 to 45% reduced carbohydrate release by 30% after 3 h and by 5% after 54 h of hydrolysis (Grabber et al., 1998a). Increased dimerization of ferulate reduced the initial hydrolysis of all neutral and acidic sugars from walls, suggesting that xylan cross-linking reduced the access of hydrolytic enzymes to all structural polysaccharides. Although diferulate cross-linking did not restrict the extent of cellulose and pectin hydrolysis, xylans with a high degree of diferulate cross-linking accumulated in the indigestible residue.

In subsequent studies (Grabber et al., 1998c), nonlignified maize walls from normal or 2-aminoindan-2-phosphonic acid treated cell suspensions, with 16.9 or 4.7 g kg^{-1} of ferulates, were artificially lignified with coniferyl alcohol to form DHP-CWs with 15.9 or 4.5 g kg^{-1} of ferulates incorporated into lignin. This reduction in ferulate-lignin cross-linking dramatically increased the initial and, to a lesser degree, the final extent of cell wall hydrolysis (Table 1). A comparable enhancement in degradability was obtained when cross-linking was reduced by methylating ferulates with diazomethane to block coupling of ferulate with monolignols during DHP-CW formation. On the basis of monosaccharide analysis, reduced cross-linking enhanced the hydrolysis of xylans and, to a lesser degree, cellulose and pectins from cell walls, which would be expected since ferulates are esterified to xylans.

These findings support the contention that selection or genetic engineering of grasses to reduce ferulate cross-linking would enhance the enzymatic hydrolysis and subsequent utilization of structural polysaccharides for nutritional and industrial purposes (Jung and Ralph, 1990). The benefits of reducing ferulate-lignin cross-linking for improving structural carbohydrate hydrolysis have been bolstered by more recent findings that direct plant selection for low levels of ferulate ether-linked to lignin improved bromegrass cell wall fermentation by rumen microorganisms (Casler and Jung, 1999). Further studies with nonlignified walls and DHP-CWs revealed that the inhibitory effects of diferulate cross-linking on wall hydrolysis can be overcome, in part, if enzyme cocktails contain high xylanase activity (Grabber et al., 1998c). Although feruloyl esterases can cleave diferulate cross-links in soluble xylans or simple model substrates (Faulds et al., 2003; Garcia-Conesa et al., 1999a), there is no evidence that these enzymes break a significant proportion of cross-links *within* lignified cell walls; therefore, they are currently of limited value for improving cell wall degradability. At present, it appears that breeding or engineering of grasses for low levels of ferulate cross-linking or developing potent microbial xylanases are more promising than feruloyl esterase treatments for enhancing forage cell wall digestion.

Benzyl Ether and Ester Cross-Linking

Numerous studies with model compounds and woody species indicate that cell walls are cross-linked by the reaction of matrix components with lignin quinone methide intermediates which are formed by β–*O*–4 coupling of monolignols (Koshijima and Watanabe, 2003). Under acidic conditions (pH $<$ 5), quinone methide intermediates react with water, uronic acids, amino acids, or neutral sugars to form relatively linear lignins substituted with α-hydroxyl groups or cross-linked by benzyl α-ester and α-ether linkages to cell wall polysaccharides and proteins. Less acidic conditions (pH $>$ 5) depress the addition of water and the formation of cross-links, while favoring the reaction of quinone methide intermediates with phenols from monolignols (or their coupling products) to form lignins with greater branching (Brunow et al., 1989; Quideau and Ralph, 1994; Sipilä and Brunow, 1991). The reaction of cell wall constituents with quinone methide intermediates at acidic pH is enhanced under hydrophobic conditions (Kishimoto et al., 2002; Sipilä and Brunow, 1991; Toikka and Brunow, 1999), indicating that benzyl ether and ester cross-links are primarily formed at the latter stages of lignification as water is excluded from the cell wall matrix. The pH of the apoplast in non-lignified tissues varies from about 4 to 7, but most often pH values between 5 and 6.5 are reported (Grignon and Sentenac, 1991). To our knowledge, no studies have measured the apoplastic pH of lignifying tissues. Structural studies of lignins and model compounds suggest that the apoplastic pH of lignifying tissues in deciduous trees may be at or below 4 (Brunow et al., 1989).

Although benzyl ester and ether cross-linking has been widely investigated in regards to lignin-carbohydrate complex formation and delignification of woody species, little is known about their influence on cell wall degradability because the abundance of these cross-links in forage and woody species is not known, and there is currently no method for directly measuring them on a routine basis. In several model studies, hydroxypropylation, ammoniation, or treatment with reducing agents lessened the inhibitory effect of lignin on cellulose or cell wall hydrolysis by rumen microflora (Sewalt et al., 1997; Sewalt et al., 1996a; Sewalt et al., 1996b). This response was in part attributed to reductions in quinone-methide mediated cross-linking but the abundance of benzyl ether or ester cross-links was not actually determined in these studies.

In our studies (Grabber et al., 2003), nonlignified walls of maize walls were artificially lignified with coniferyl alcohol at pH 4 or 5.5 to vary the propensity of quinone methide intermediates to couple with cell wall constituents (Fig. 2). Incorporation of wall-bound polymers at pH 4 was 7 to 54% less than at pH 5.5 because of more rapid inactivation of cell wall peroxidase under acidic conditions. Adjusting data to account for differences in lignin content revealed that forming complexes at a pH of 5.5 instead of 4.0 increased the release of sugars from cell walls (Table 1). As expected for cross-links formed via quinone methides, lignification under acidic pH conditions depressed wall degradability only as cell walls become hydrophobic with at least moderate amounts of lignin. It was also observed that the pH-related changes in degradability noted with coniferyl alcohol DHP-CWs did not occur with DHP-CWs formed with coniferaldehyde, a structurally related monolignol precursor that does not permit the addition of nucleophiles to quinone-methide intermediates (Connors et al., 1970). Finally, the differential effect of lignification pH on degradability was not related to ferulate cross-linking because all treatments had comparable amounts of ferulate monomers and dimers incorporated into lignin. Overall, these results indicated that poor degradability of DHP-CWs formed under low pH was related to enhanced benzyl ester and ether cross-linking in cell walls. Additional studies are, however, needed to confirm the existence of these cross-links in our model system and to assess whether acidic pH increases their abundance in cell walls.

Potential cross-linking of pectin to lignin via quinone methide intermediates was also investigated by treating nonlignified walls with pectin methyl esterase (PME) before DHP-CW formation at pH 4 (unpublished data, 2000 and 2003). In this study, cell walls contained 100 g kg^{-1} of uronic acids, 57% of which were methyl esterified. Methyl esterification of uronosyls would prevent their addition to lignin quinone methide intermediates (Fig. 2). Removal of methyl groups by PME should therefore enhance benzyl ester cross-linking between uronosyls and lignin and restrict the degradation of pectin. In these studies, a 64% reduction in methylation by PME treatment reduced pectin degradation in DHP-CWs by 55% after 4 h and by 7% after 72 h of enzymatic hydrolysis. In contrast, a reduction of uronosyl methylation slightly enhanced the degradation of pectins in non-lignified walls. Variations in pectin methylation had lit-

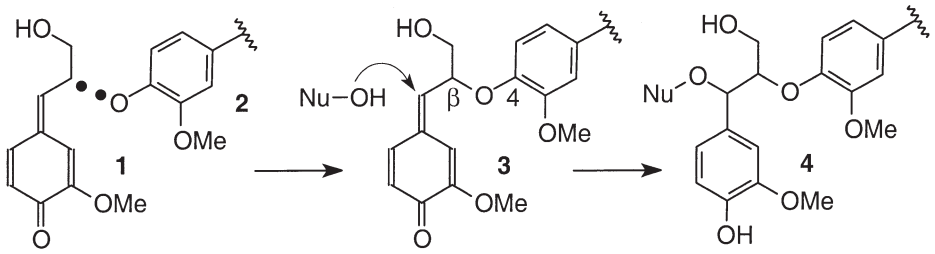

Fig. 2. Monolignol (1) and lignin (2) radicals can undergo β-*O*-4 coupling to form quinone methide intermediates (3). These intermediates are stabilized by the addition of nucleophiles (Nu), such as water, uronic acids, or neutral sugars to form structures (4), which are substituted with α-hydroxyl groups or cross-linked to the cell wall matrix by benzyl α-ester or benzyl α-ether linkages.

tle effect on the release of total sugars from DHP-CWs, indicating that the ease of pectin hydrolysis had little impact on the hydrolysis of other structural polysaccharides. These studies clearly implicate benzyl-uronate cross-links as a factor limiting pectin degradation but additional studies are needed to confirm the existence of these cross-links in our model system and to assess whether variations in pectin methylation influence their abundance in cell walls.

Prospects for Developing a DHP-CW System for Dicots

Although primary walled DHP-CWs are a suitable model for lignified grass cell walls, the converse is true in dicots, where secondary walls of xylary tissues are much more heavily lignified and resistant to degradation than primary and other secondary walled tissues (Engels and Jung, 1998; Grabber et al., 2002b). The makeup of noncellulosic polysaccharides also differs considerably between primary and secondary walls in dicots (Grabber et al., 2002b; Wilson et al., 1989). Because of their interactions with lignin, such variations in noncellulosic polysaccharides may modulate how lignin limits cell wall degradability in dicot tissues. Cellulose structure and deposition patterns also differ between primary and secondary walls in dicots, as well as grasses, but this is less of a concern in model studies because cellulose and lignin do not directly interact within cell walls. As a result, DHP-CW modeling of lignified dicot cell walls should be done with primary and secondary walled tissues.

Although a variety of undifferentiated dicot cell cultures could be used as a source of primary walls for forming DHP-CWs, modeling secondary wall lignification would be more complex because differentiating cultures, like plants, produce a mixture of nonlignified primary and lignified secondary-walled cell types. Therefore, cell cultures or plants must be grown with lignification inhibitors and then subjected to tissue fractionation if defined cell types are to be artificially lignified. Since lignification occurs almost concurrently with secondary wall formation (Blee et al., 2001; Taylor and Haigler, 1993; Terashima et al., 1993), it is not possible to fully mimic this process with the DHP-CW system because cell walls must be isolated before artificial lignification. Despite these limitations, our group is pursuing the development of a DHP-CW system for dicots because our understanding of lignin-matrix interactions and cell wall degradability in dicots is quite rudimentary compared with what has been discovered by model studies of grass cell walls.

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